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13. ABSTRACT (Maximum 200 Words) The AIB1 protein is a member of a coactivator family that potentiates the transcriptional activity of the nuclear hormone receptors. AIB1, is amplified in certain breast and ovarian cancers and has been suggested that AIB1 plays a causative role in breast cancer development. Our lab recently identified AIB1 as a target of the MAPK signaling pathway (Font de Mora and Brown, Mol Cell Biol 20:5041, 2000). Based on these findings, we propose that the phosphorylation of AIB1 by MAPK may represent part of the molecular mechanism that integrates signals from steroid hormones and growth factors. In order to identify the sites of AIB1 that are phosphorylated by MAPK, seven potential phosphorylation sites were targeted for point mutations and or deletions. Previously we showed by an In vitro phosphorylation assay by (Erk2) that three out of the seven putative phosphorylation sites tested were most likely to be the specific MAPK phosphorylation sites. In order to investigate the physiological relevance of the AIB1 phosphorylation sites, we subcloned all mutants into GAL4DBD vector. We found that those mutants that showed low phosphorylation <i>in vitro</i> , gave the lowest transactivation levels when tested <i>in vivo</i> . In addition we were able to differentiate the sub-cellular localization of AIB1 after phosphorylation by performing transient transfection in COS cells using a constitutively active MAPK vector and a kinase inhibitor vector and further on performing immunofluorescence.				
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Introduction

Our laboratory has been interested in unraveling the cellular and molecular mechanisms underlying estrogen responses in both normal and neoplastic tissues. Estrogens have central roles in the control of development, sexual behavior, and reproductive functions. Their effects have also been linked to the progression of a majority of human breast cancers. The diverse biological effects of estrogens are mediated by the estrogen receptors (ER), which are members of the nuclear receptor (NR) superfamily¹. ER also associates with different classes of coregulatory factors that have either coactivator or corepressor function. The coactivators potentiate transcriptional activation by ER in an agonist-, but not antagonist-dependent manner. In general, these coactivators can be divided into five families: (1) the p160 family of proteins, including SRC-1/ERAP160/NCoA-1^{2,3}, TIF2/GRIP1/NCoA-2⁴⁻⁶, and p/CIP/ACTR/AIB1/RAC3⁷⁻¹⁰; (2) the non-p160 members, such as ARA, RIP140, TIF1 and Trip/SUG1¹¹⁻¹⁴; (3) the cointegrator CBP/p300 and its associating protein p/CAF^{15,16}; (4) the distinct DRIP/TRAP/ARC complex¹⁷⁻¹⁹; and (5) the ATP-binding regulators, such as hBrm and BRG-1^{20,21}. With identification of these coactivators, the central question by which mechanisms these multiple factors regulate ER activity remains.

AIB1 (Amplified in Breast Cancer) was discovered as a gene that is amplified in certain breast and ovarian cancers^{9,10}. AIB1 is a member of the p160 family of ER coactivators and our lab recently identified AIB1 as a target of the MAPK signaling pathway²². This signaling pathway is triggered, for example, by growth factors of the insulin-like growth factor (IGF) and epidermal growth factor (EGF) family. These growth factors and their receptors have also been implicated in the development and progression of breast tumors. We propose that the phosphorylation of AIB1 by MAPK may represent part of the molecular mechanism that integrates signals from steroid hormones and growth factors. Furthermore, we hypothesize that AIB1 phosphorylation may contribute to the role that AIB1 plays in the development of breast cancer.

The goals for this proposal were to identify the sites in AIB1 that are phosphorylated by MAPK *in vitro* and *in vivo* and to determine the importance of these phosphorylation sites for the function of AIB1 as a transcriptional activator. The *in vitro* mapping of the phosphorylation has been described in the last report. In brief, I demonstrated that *in vitro* phosphorylation of AIB1 by MAPK is restricted to the regulatory domain of AIB1. This result was shown using *in vitro* phosphorylation of two AIB1 fragments. The first fragment, F12 (amino acid 578-1131), contains both the regulatory domain (RD), where the interaction with ER takes place, and the activation domain 1 (AD1). The second fragment, F3 (amino acid 980-1131), only contains AD1. AD1 has been shown previously to function through the recruitment of p300/CBP (7). *In vitro* phosphorylation of bacterially expressed Gst-AIB1 fusion proteins showed that fragment 12 was strongly phosphorylated by the MAPK family member erk-2. In contrast, fragment 3 showed very low or no phosphorylation. Based on this result, the amino acid sequence of fragment 12 was examined and 7 consensus phosphorylation sites for MAP kinase (PXX (S/T) P) were identified. Point mutations of these sites were generated as serine to alanine mutations. In addition to the point mutations, three internal deletions were generated to eliminate several phosphorylation sites at the same time (see diagram in previous report). Using the same *in vitro* phosphorylation assay, I was able to determine that three of these seven putative phosphorylation sites (sites 728, 867, and 915) seemed to be the preferred sites for phosphorylation by MAPK, *in vitro*.

BODY

Task 2: To determine the relevance of the MAPK phosphorylation sites mapped in *Task 1* for the function of AIB1 as a transcriptional activator (months 6-12). **Completed.**

Task 2 Schedule: *Task 2* was accomplished between months 13-18. It was accomplished 6 months behind schedule, due to delays in *Task 1* because of experimental difficulties. *Task 2* took 6 months to finish, as anticipated in the SOW.

Task 2 Results: Stimulation of AIB1 transactivation function by MAPK *in vivo* requires serines at positions 728 and 915.

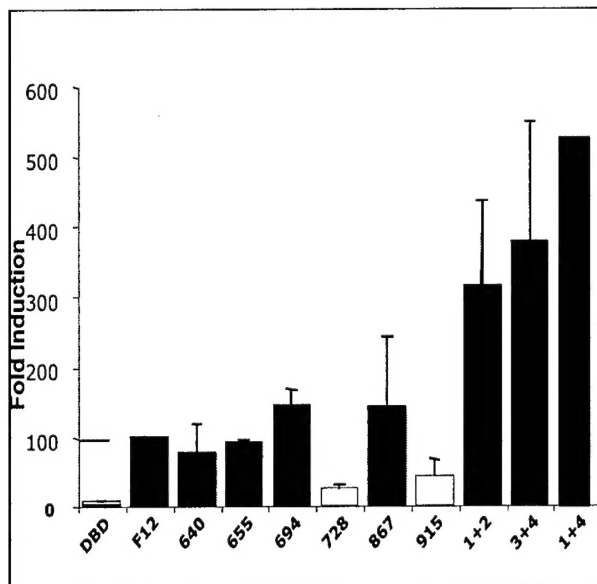
In order to test the AIB1 constructs containing mutated putative MAPK phosphorylation sites for their transactivation abilities the wild type AIB1 and mutations were generated as fusion constructs fused to the Gal4 DNA binding domain (Gal4 DBD). This strategy provided the AIB fragments with a DNA binding domain in order to test for AIB1 transactivator function independent of the estrogen receptor. This strategy was chosen because transactivation by the estrogen receptor itself can be modulated by MAPK. The Gal4 DBD-AIB1 fusion genes were then tested in reporter assays as to which phosphorylation sites were required for MAPK modulation of AIB1 coactivator function. The region of AIB1 analyzed by this assay was the region between amino acids 578 and 1131 (termed fragment 12). This region has been shown to be the target of phosphorylation by MAPK, *in vitro*, as shown in *Task 1* (see last report) and at the same time to show the strongest induction of transactivation following stimulation by MAPK, *in vivo*, as previously demonstrated in our lab (22).

The mutant Gal4 DBD-AIB1 fragment 12 constructs were generated by subcloning the respective mutations of the *gst*-AIB1 fusion genes used for the *in vitro* phosphorylation studies done in *Task 1*. This strategy eliminated the need to recreate the mutations in another vector. All seven mutants, plus the three deletion mutants created in *Task 1* were amplified by PCR, thereby attaching restriction sites *Bgl*III and *Nhe*I to the 5' and 3' end, respectively. These restriction sites allowed directional subcloning into the pCMX Gal4-DBD vector, cut with *Bam*HI and *Nhe*I, in frame with the Gal4 DBD. The expression constructs were sequenced and transcribed and translated *in vitro* to ensure integrity of the sequence and expression, respectively.

The Gal4 DBD AIB1 fusion genes containing the various mutations were then tested for transcriptional activity. This was done by transiently co-transfecting COS cells along with a reporter plasmid. This plasmid contained the GAL4 DNA binding site fused to a minimal promoter from the Herpes Simplex thymidine kinase gene (*tk*) driving the transcription of a luciferase reporter gene. The transfections included a *tk-lacZ* plasmid as an internal control. To determine the contribution of the MAPK pathway to AIB1 activity, GAL4-AIB1 was cotransfected together with MAPK phosphatase-1 (MKP1), to inhibit all endogenous MAPK activity, or together with a constitutively active mutant, MEK1 (RΔF), stimulating the MAPK pathway. This assay is analogous to the assay published previously that established that AIB1 coactivator function can be enhanced by MAPK (22).

In the absence of either MKP1 or RΔF, Gal4-AIB1 fragment 12 exhibited potent transcriptional activation, as expected (not shown). Cotransfection of MKP1 reduced the level of activation stimulated by Gal4-AIB1, while cotransfection of RΔF substantially augmented Gal4-AIB1-mediated transactivation of wt AIB1 F12, as previously reported (22). For each mutant construct, the induction of AIB1 transactivation by MAPK was calculated as a ratio of the luciferase activities of the transfections with the constitutively active MEK1 and the MAPK inhibitor, MKP1 (Figure 1). While the pCMX Gal4 DBD vector alone (DBD) did not exhibit any response to MAPK, AIB1 fragment 12 without internal mutations (F12) showed about 100 fold induction, in agreement with our previously published data (22). Point mutations 640, 655 and 694 also showed ~ 100 – 150 fold induction and thus did not differ significantly from wt fragment 12 (F12). In sharp contrast, point mutations 728 and 915

Figure 1. Induction of luciferase activity in COS cells co-transfected with AIB1 mutants and RΔF or MKP1



significantly reduced the ability of AIB1 fragment 12 to be induced by MAPK. The results from point mutation 867 are somewhat inconclusive, due to the size of the error bars. More data need to be gathered for this mutant to allow a conclusion.

Surprisingly, MAPK induction of internal deletion of larger portions of AIB1 containing several putative MAPK sites (constructs 1+2, 3+4, and 1+4) was substantially enhanced as compared to wt fragment 12. Presumably the deletion of larger portions of the regulatory domain led to this effect. It has been previously shown that the regulatory domain exerts a negative effect on AIB1 transcriptional activation that may be abolished when larger internal portions of this domain are deleted. Thus, the internal deletions do not allow to draw any conclusions regarding the combinatorial effect of mutating several MAPK sites. My data indicate that both sites, serine 728 and serine 915, are required for optimal induction by MAPK.

Together, these data show convincingly that MAPK mediates its effect on AIB1 transactivation in a direct manner through specific sites that are MAPK consensus sites. The two sites, positions 728 and 915, mapped as MAPK responsive sites in the functional assay, were previously shown to be the major sites phosphorylated *in vitro* (see last report and this report, introduction). Thus, the *in vivo* data from this report are consistent with the *in vitro* data from the previous report.

Task 3: To examine the biochemical changes that occur in the multisubunit coactivator complex after AIB1 phosphorylation, *in vivo* (months 12-18). **Initiated.**

Task 3 Schedule: Task 3 was initiated behind schedule, due to delays in Task 2. The design of the experiments required some modifications and now utilizes mutants generated according to the results of Task 2.

In the original experimental design MCF-7 cells were to be treated with a MAPK activator or inhibitor, followed by analysis of the transcription complexes formed at specific ER target genes using a chromatin immunoprecipitation (ChIP) assay. Specifically, the presence of ER, AIB1, and CBP/p300 and p/CAF in the transcription complex were to be analyzed. However, we realized that in this experimental design the interpretation of results would be complicated by the fact that ER itself is a target of MAPK and the transactivation capacity of ER itself is modulated by MAPK.

Thus, we have slightly modified the experimental design by using transfected MCF-7 cells instead of untransfected cells. MCF-7 cells will be transfected with myc-tagged, wild type or mutated full length AIB1 constructs. Specifically, we have constructed full length AIB1 with serine to alanine mutations at positions 728 and 915, as well as a double mutant. All mutated constructs, as well as the wild type construct, were made containing in the vector pcDNA3.1-myc, providing all constructs with an N terminal myc tag.

We are in the process of transfecting these constructs into MCF-7 cells in conjunction with constructs expressing a constitutive activator or a dominant negative inhibitor of the MAPK pathway (MEK1-RΔF and MAPK phosphatase-1, respectively). The latter constructs are the same as used in Task 2. The

follow up analysis of the experiment will be as described in the SOW, by performing chromatin immunoprecipitation (ChIP) assays. Instead of an antibody against AIB1 an antibody against the myc tag will be used. This will restrict the analysis to complexes containing the transfected AIB1 and AIB1 mutants, as opposed to endogenous AIB1. We will specifically analyze MAPK-mediated differences in the association between AIB1 and ER as well as AIB1 and CBP/p300 or p/CAF. We will also analyze changes in levels of ER target genes, as previously outlined. Differences between wt and mutated AIB1 will directly demonstrate involvement of MAPK and of the mapped phosphorylation sites in the association of AIB1 with ER, CBP/p300, and/or pCAF, and the effect of MAPK-mediated AIB1 phosphorylation in the transcription of target genes.

As a first step for these experiments, we have already tested the myc-tagged, full length, wild type AIB1 construct in transfections for expression in COS cells and in 293T cells. A western blot of protein extracts probed with an anti myc-tag antibody and an anti AIB1 antibody showed a band with the expected size for AIB1. We have also performed transfections of wild type AIB1 in conjunction with the constructs activating or repressing MAPK (MEK1-RΔF and MKP-1, respectively). Because of initial difficulties transfecting MCF-7 cells, we used COS and 293T cells to ensure that the constructs were expressing correctly. We also analyzed some of the transfected cells by immunofluorescence, using an anti myc tag antibody, to obtain an estimate of the transfection efficiency that we were achieving. When doing this, we observed that the transfected (myc-tagged) AIB1 changed its intracellular localization depending on the presence of MAPK activity. In the presence of active MAPK, AIB1 yielded a distinct, punctual staining within the nucleus that appeared to be limited to the nucleoli. This was in contrast with cells that were transfected with AIB1 alone or together with MKP1, which resulted in a diffuse nuclear staining for AIB1. The localization of AIB1 in the nucleoli after MAPK phosphorylation could indicate that the cells are cycling more actively since the presence of multiple nucleoli is characteristic of telophase in mitotic cells.

Through this observation we have defined another important property of AIB1 that is subject to modification by MAPK, namely, its ability to change its intracellular location. At the same time this observation provides an independent readout, besides the function of AIB1 as a transcriptional coactivator, for the actions of MAPK on AIB1, which are the subject of this investigation. We therefore plan to analyze the effect of MAPK on the intracellular localization of the above-described AIB1 mutants lacking the mapped MAPK phosphorylation sites. If we can show that the mutations are no longer subject to regulation by MAPK, as we expect, this would substantiate any data obtained when measuring their ability to act as ER coactivators in a ChIP assay as proposed in the experiments for *Task 3*. At the same time this result provides an opportunity to investigate the effect of MAPK on AIB1 intracellular translocation, a phenomenon not described before.

Task 4: To examine the role of AIB1 phosphorylation in the regulation of ER dependent genes (months 18-36). **Initiated.**

Task 4Schedule: *Task 4* was initiated on schedule, coincidentally with *Task 3*, in order to compensate for the delays in *Task 1*.

Task 4a It will be investigated whether AIB1 phosphorylation mutants can act in a dominant negative fashion to repress transcription of ER dependent genes and to decrease proliferation of breast cancer cell lines.

As outlined in the SOW, the phosphorylation mutants determined by *Tasks 1 and 2* to contain the *in vitro* and *in vivo* MAPK phosphorylation sites were constructed as full length cDNAs, without the Gal4 DBD, but provided with an epitope tag. Instead of the T7 tag proposed in the SOW, we chose to use a myc tag, because of the availability of vectors, antibodies, protocols and expertise in our department. Of note, we are now also using these constructs for *Task 3*, as described above. The

myc-tagged wild type construct has already been tested for expression (see *Task 3*) in COS and 293T cells. Transfection of MCF-7 cells, as proposed in SOW4, is underway.

Before performing cell cycle analysis on transfected MCF-7 cells, we wanted to establish whether the high levels of AIB1 detected in MCF-7 cells did contribute to survival and/or proliferation of this cell line. To address this question, we used RNA interference to downregulate AIB1 expression in MCF-7 cells and subsequently analyze the cells by FACS for cell cycling. RNA interference was performed by transfecting MCF-7 cells with ribooligonucleotides complementary to AIB1 mRNA sequences. Knockdown of AIB1 expression was monitored by western blot and amounted to a ~ 80% decrease in AIB1 expression. Cell cycle analysis was performed by FACS after propidium iodide staining, as proposed in the SOW. Decrease in AIB1 expression resulted in a 40% increase in apoptosis, directly demonstrating that MCF-7 cells depend on AIB1 for survival. After establishing this important fact, we are now in the position to analyze whether AIB1 requires MAPK phosphorylation to mediate survival of MCF-7 cells.

Task 4b AIB1-containing transcription complexes in the transfected cells will be analysed by ChIP assay, using an anti T7 antibody to selectively precipitate transcription complexes containing the transfected wt or mutant AIB1, but not the endogenous AIB1.

This part of *Task 4* is identical to the modified *Task 3* and has been initiated (see *Task 3*). As stated above, instead of a T7 tag and anti T7 antibody, we are now using a myc tag and anti myc antibody.

Training accomplishments

Training was obtained in the following techniques and methods: FACS analysis, immunohistochemistry, immunofluorescence, Real Time PCR, chromatin immunoprecipitation (ChIP) assays, RNA interference, microarrays, Genespring software for microarray analysis.

Key Research Accomplishments

- Modulation of AIB1 transactivator function by MAPK was found to require serines at positions 728 and 915.
- *In vivo* phosphorylation sites on AIB1 were found to be identical to *in vitro* phosphorylation sites.
- Downregulation of AIB1 expression in MCF-7 cells was found to increase the incidence of apoptosis.

Reportable Outcomes

Regulation of the Activation of AIB1, an Estrogen Coactivator, by Growth Factor Signals. Abstract presented at the 2002 Era of Hope DOD breast cancer research meeting Sep. 2002

AIB1 Phosphorylation Sites Targeted by MAPK. Abstract to be presented at the 2004 Keystone Symposium: Nuclear Receptors (J8), Feb.28 – Mar. 4, 2004.

Conclusions

We have completed the part of the project that was aimed at identifying the target sites on AIB1 phosphorylated by MAPK in vivo and in vitro. We have demonstrated that MAPK phosphorylation of these sites results in an increase in AIB1-mediated transactivation. We are now in the process of analyzing what role AIB1 phosphorylation by MAPK plays in a) the formation of the estrogen receptor/ AIB1/ CBP/p300 transactivation complex (*Task 3*), b) the regulation of estrogen receptor target genes (*Task 4*), c) the regulation of cell survival and apoptosis (*Task 4*), and d) the regulation of AIB1 intracellular localization.

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Appendix Cover Sheet

**REGULATION OF THE ACTIVITY OF AIB1,
AN ESTROGEN COACTIVATOR,
BY GROWTH FACTOR SIGNALS**

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AIB1 (Amplified in Breast Cancer) was discovered as a gene that is amplified in certain breast and ovarian cancers. AIB1 is a member of the p160 family of steroid receptor coactivators.

Our lab recently identified AIB1 as a target of the MAPK signaling pathway (Font de Mora and Brown, Mol Cell Biol 20:5041, 2000). This signaling pathway is triggered, for example, by growth factors of the insulin-like growth factor (IGF) and epidermal growth factor (EGF) family. These growth factors and their receptors have also been implicated in the development and progression of breast tumors. We propose that the phosphorylation of AIB1 by MAPK may represent part of the molecular mechanism that integrates signals from steroid hormones and growth factors. Furthermore, we hypothesize that AIB1 phosphorylation may contribute to the role that AIB1 plays in the development of breast cancer.

The first goals for this proposal are to identify the sites in AIB1 that are phosphorylated by MAPK *in vitro* and *in vivo* and to determine the importance of these phosphorylation sites for the function of AIB1 as a transcriptional activator.

In order to map the regions and define the precise amino acids of AIB1 that are phosphorylated by MAPK *in vitro*, we made deletion and point mutants of AIB1. The mutants were expressed as GST fusion proteins, phosphorylated with ³²Pγ-ATP and recombinant MAPK, *in vitro*, and analyzed by SDS gel electrophoresis and autoradiography. We found that most of the AIB1 phosphorylation by MAPK occurs within the regulatory domain of AIB1, which is also required for the maximal induction of AIB1 coactivator function by MAPK, as shown previously in our lab. This region contains 7 putative MAPK consensus sites (PXX(S/T)). Our results indicate that 4 of these sites are phosphorylated to a similar degree while 3 of them are clearly far less phosphorylated. A functional analysis of these mutations as GAL4 fusion constructs *in vivo* in cell lines should be able to further narrow down the relevant phosphorylation sites.

Our study will help to understand how AIB1 is modulated by MAPK. This may help to determine how AIB1 may be involved in the development of breast cancer, which is the most common cancer among women and the second leading cause of cancer deaths among women in U.S.A.

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Abstract presented in the 2004 Keystone symposium

Feb 28 - Mar 4, 2004

Nuclear Receptors: Steroid Sisters (J8)

Nuclear Receptors: Orphan Brothers (J7)

Keystone, Colorado

AIB1 Phosphorylation Sites Targeted by MAPK

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Amplified in breast cancer 1 (AIB1) has been identified as a gene that is preferentially amplified in estrogen receptor (ER)-positive breast cancers and acts as a ligand-dependent nuclear receptor coactivator. We previously demonstrated that AIB1 can be phosphorylated by MAPK, which enhances the transcriptional activity of AIB1. This suggests that the ability of growth factors to modulate ER activity in a ligand-independent manner through the MAPK signaling pathway may occur through activation of AIB1. In order to map the AIB1 domain that is involved in phosphorylation by MAPK, several AIB1 fragments were generated as GST fusion proteins in the vector pGEX-4T-1. Using an in vitro phosphorylation assay we found that AIB1 phosphorylation by the MAPK Erk2 is restricted to the region containing the regulatory domain (RD). This region was examined for the presence of consensus MAPK phosphorylation sites (PXX(S/T)P), and 7 potential sites were identified. AIB1 constructs with point mutations or deletions at each of these sites were generated, and these mutated or deleted AIB1 constructs were linked to the Gal4 DNA binding domain, and then transiently transfected into COS cells along with a luciferase reporter regulated by the Gal4 response element. Cells were untreated or were treated with a MAPK activator or inhibitor, and alterations in luciferase activity were monitored. These experiments allowed the identification of specific functionally important sites within the RD region of AIB1 that are directly targeted by Erk2.

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